

Massive bowel resection upregulates intestinal expressions of *Rbp2* and *Apoa4* mRNAs and alters intestinal vitamin A status in rats

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ABSTRACT

Short bowel (SB) syndrome causes the malabsorption of various nutrients. Among them, vitamin A is important for many physiological activities. Vitamin A is taken up by absorptive epithelial cells of the small intestine and discharged into lymphatics as a component of chylomicrons and delivered to the liver. Here, we used a rat model of SB syndrome to assess its effects on the expression of genes associated with the absorption, transport and metabolism of vitamin A. In SB rats, intestinal expression levels of mRNAs for cellular retinol-binding protein II (CRBP II, gene symbol *Rbp2*) and apolipoprotein A-IV (gene symbol *Apoa4*) were higher than in shams. Absorptive epithelial cells stained positively for both CRBP II and lecithin:retinol acyltransferase, both needed for effective esterification of vitamin A. In SB rats, the ileal retinol content and the jejunal retinyl esters content were lower than in sham rats. These results suggest that the elevated expression levels of *Rbp2* and *Apoa4* mRNAs in SB rats contribute to the effective esterification and transport of vitamin A.

Introduction

Short bowel (SB) syndrome is the consequences of malabsorption from an intestinal surface area insufficient to maintain growth and normal nutritional status (1,2). Intestinal adaptation is a well-known phenomenon that increases absorptive capacity after surgical resection of the small intestine. The most prominent feature of intestinal adaptation is the proliferation of intestinal cells that increases crypt depth and enlarges the length and width of the villi (3). It is known that micronutrients affect several aspects of intestinal adaptation. Wang *et al.* (4) reported that intestinal adaptation was facilitated by retinoic acid, an active form of vitamin A. Intravenous administration of retinoic acid has trophic effects in SB rats by inhibiting apoptosis and stimulating crypt cell proliferation.

Vitamin A is a fat-soluble vitamin needed for many physiological activities. Retinyl esters (REs), animal-derived forms of vitamin A, are hydrolyzed to retinol before entering the absorptive epithelial cells. In contrast, β -carotene, a vegetable-derived form of vitamin A, is converted to retinal after entering the absorptive epithelial cells and is then reduced to retinol within the cells (5). The retinol thus produced binds to cellular retinol-binding protein II (CRBP II, gene symbol *Rbp2*) in absorptive epithelial cells, forming a retinol-CRBP II complex. This complex is esterified to REs by lecithin:retinol acyltransferase (LRAT, gene symbol *Lrat*). REs are then discharged basolaterally into lymphatics as a component of chylomicrons and delivered to the liver via general circulation (6). In hepatocytes, REs are hydrolyzed to retinol and complexed with retinol-binding protein (RBP, gene symbol *Rbp4*). The retinol-RBP complex is transferred to hepatic stellate cells (HSCs) where the retinol binds to CRBP I (gene symbol *Rbp1*) and is again esterified to REs by LRAT for storage (7,8).

CRBP I and II are members of the retinoid-binding protein family (9) which constitutes the calycin protein superfamily together with the fatty acid-binding proteins (FABPs) (10). In mammals, CRBP I is expressed ubiquitously (11) whereas CRBP II is expressed specifically in the intestine and the fetal liver (12). CRBP II increases perinatally, changes that are associated with growth and differentiation of the intestine (13). CRBP II knock-out mice were more susceptible to vitamin A deficiency, implying that CRBP II plays an important role in vitamin A homeostasis (14). Takase *et al.* reported that jejunal-bypass surgery led to a

marked increase in the amounts of CRBP II in the residual jejunal segment of rats (15). The upregulation of the *Rbp2* gene in SB rats was also reported by Dodson *et al.* (16). They showed that upregulation of *Rbp2* mRNA resulted in growth of absorptive epithelial cells, presumably through increased vitamin A absorption by those cells (17).

As noted above, vitamin A is thought to be trophic to the bowel, may therefore be important during adaptation in SB syndrome, and deficiency of vitamin A may have negative consequences for adaptation. Here, we examined the impact of significant bowel resection on vitamin A absorption, transport and metabolism. Towards that ends, we used a rat model of SB and analyzed vitamin A-related factors by quantitative RT-PCR, Western blotting and immunohistochemistry as well as measurements of vitamin A in tissues and cultured cells.

Materials and methods

Animals and surgical procedures. Protocols for animal experimentation were approved by the Animal Research Committee, Akita University Graduate School of Medicine. All animal experiments adhered to the “Guidelines for Animal Experimentation” of the University. Male Sprague-Dawley rats (ages ranged from 8 to 11 weeks, 280 to 480 g body weight) were used for this study. Twelve h after fasting, each animal was anesthetized. In sham rats, the gut was simply divided ten cm proximal to the ileocecal junction and anastomosed without resecting any part (Fig. 1A). In SB animals, the small bowel was resected from a point five cm distal to the ligament of Treitz to a point ten cm proximal to the ileocecal junction, resulting in a 75% resection of the small intestine. Mesenteric vessels were ligated with sutures, and bowel continuity was restored by end-to-end anastomosis (Fig. 1B, arrow). Rats fasted for 24 h but were allowed free access to water. Short bowel and sham rats were then fed with Elental (Ajinomoto Pharmaceuticals, Tokyo, Japan) according to the manufacturer’s instructions. On day seven, animals were sacrificed after intraperitoneal injection of pentobarbital, and livers and small intestines were excised and immersed in 10% formalin. For RNA preparation, small pieces of livers and small intestines were quick-frozen in liquid nitrogen and kept frozen at -85°C.

A pair of rats underwent surgery for each trial: one animal for SB and one for the sham operation. From 19 pairs of rats, 13 SB rats and 14 shams completed the trial. The jejuna of sham rats were collected from 8 animals out of 14 animals that underwent operation. Over the first two days following surgery, both SB and sham rats lost weight (Fig. 1C). Afterwards, the sham rats gradually regained weight, while SB rats continued to lose weight.

RNA extraction and quantitative RT-PCR. Frozen rat livers and intestines were dissolved in Trizol reagent (Life Technologies, Carlsbad, CA) and total RNA was isolated. The cDNAs were synthesized from five µg of total RNA using SuperScript III Reverse Transcriptase (Life Technologies) using an oligo(dT)₁₈ primer. Quantification of cDNA was done with LightCycler 480 SYBR Green I Master (Roche Diagnostics, Meylan, France). Transcript amounts were normalized against the *Gapdh* transcript. The nucleotide sequences used are

summarized in Table 1.

Organelle fractionation. Small pieces of tissues (50 to 150 mg) were homogenized in 0.5 ml of cell disruption buffer (0.25 M sucrose, 20 mM HEPES, pH 7.5) using a Polytron CT 2100 homogenizer (Kinematica AG, Littau/Luzern, Switzerland). Samples were further homogenized by 20 strokes with a Dounce tissue grinder (Wheaton, Millville, NJ). Homogenates were centrifuged for seven min at $1,100 \times g$. Supernatants were further centrifuged at $1,900 \times g$ for 30 min. Pellets were set aside as mitochondrial fractions and supernatants were again centrifuged at $105,000 \times g$ for 1 h. The pellets were set aside as microsomal fractions and final supernatants were cytosol fractions. Pelleted organelle fractions were dissolved in 100 μ l of cell disruption buffer. Protein concentrations of all organelle fractions were determined using the BCA protein assay reagent (Thermo Scientific, Rockford, IL).

Western blotting. Fifty μ g of each organelle fraction was separated by SDS-PAGE and transferred to a PVDF membrane (Atto, Tokyo, Japan). Membranes were incubated with primary antibodies against either CRBP I (1:200 dilution, sc-30106, Santa Cruz Biotechnology, Santa Cruz, CA), CRBP II (1:10 dilution, HPA035866, Sigma-Aldrich, St. Louis, MO) or LRAT (1:200 dilution, Immuno-Biological Laboratories, Gunma, Japan). A peroxidase-conjugated secondary antibody (1:5,000 dilution, 111-035-003, Jackson ImmunoResearch, West Grove, PA), was applied. Bound antibodies were detected by ECL chemiluminescence (GE Healthcare Bio-Sciences, Piscataway, NJ) and recorded on X-ray film (Fujifilm, Tokyo, Japan). As positive controls, whole cell lysates from HEK293T cells transfected with *Rbp1* or *Rbp2* plasmids were used.

Immunohistochemistry. Paraffin-embedded ilea were sectioned at 3 μ m intervals. To unmask the antigens, specimens were boiled in citrate buffer (0.01 M, pH 6.0) for 25 min. For inactivation of endogenous peroxidase activity, slides were immersed in 1% H_2O_2 for 20 min. Specimens were incubated with either CRBP II antibody (1:10 dilution) or LRAT antibody (1:100 dilution) overnight at 4°C. Slides were treated with a 1:500 dilution of peroxidase-conjugated secondary antibody and incubated with DAB substrate (Roche

Diagnostics). Nuclei were counterstained with Mayer's hematoxylin solution (Wako Pure Chemical, Osaka, Japan). Specimens were recorded digitally using NanoZoomer Digital Pathology (Hamamatsu Photonics, Hamamatsu, Japan).

Quantitative analysis of retinol and REs by HPLC. Small pieces of tissue (30 to 100 mg) were homogenized in 0.5 ml of cell disruption buffer (20 mM Tris-HCl, pH 8.0) using a Polytron CT 2100 homogenizer. After the addition of 0.5 ml methanol and 1.75 ml dichloromethane, homogenates were vigorously agitated with a Vortex mixer for one min. Then, 0.5 ml of distilled water and 2.75 ml of hexane were added and agitated with a Vortex mixer for five sec, and the samples were centrifuged for five min at $3,000 \times g$. The supernatants were set aside in a pear-shaped flask. The same procedures were carried out using the lower layer of the centrifuged samples without the addition of water. The dried supernatant was dissolved in 200 μ l of N1 (mixture of 125 ml benzene, 25 ml tert-butyl methyl ether, 0.2 ml ethanol and 350 ml hexane) and assessed by HPLC (ELITE LaChrom, HITACHI, Tokyo, Japan) with a silica gel column (SL12S03-1506WT, YMC, Kyoto, Japan) for quantitation of retinol. The flow-through fraction containing REs was collected, dried, and saponized in KOH (0.33 M in ethanol) for 30 min at 40°C. Then, 1.75 ml of dichloromethane and 2.75 ml of hexane were added to the saponized samples, and mixed for 30 sec. One ml of distilled water was added and mixed for 30 sec and centrifuged for five min at $3,000 \times g$. The supernatant was set aside in a pear-shaped flask. The same procedures were repeated. The retinol contained in the extracts was analyzed by HPLC.

Plasmids. The cDNAs for CRBP II (*Rbp2*) and LRAT (*Lrat*) were amplified from isolated rat HSCs by RT-PCR using primers designed from the reported cDNA sequences (GenBank: NM_012640 for *Rbp2* and GenBank: NM_022280 for *Lrat*) and inserted into the *KpnI* and *EcoRI* sites of pcDNA3.1 (Life Technologies). The primer pairs used for cloning of *Rbp2* and *Lrat* were 5'-GACTGGTACCATGACGAAGGACCAGAATGG-3' / 5'-GGAATTCTCACTTCTTTTTGAACACTTGTC-3' and 5'-GACGGTACCATGAAGAACTCAATGCTGGAG-3' / 5'-GACTGGAATTCCTAGCCAGACATCATCCAC-3', respectively. Forty cycles of PCR was required to

amplify the cDNA for *Rbp2* from HSCs. The cloned cDNAs were verified by sequencing.

Transfection. Transfection of plasmids into HEK293T cells was done with Lipofectamine 2000 reagent (Life Technologies). Two μg each of *Rbp2* and *Lrat* cDNAs was added to a 6-cm dish ($n = 8$ for each condition). Eighteen h after transfection, cells were treated with ten μM retinol (Sigma-Aldrich, St. Louis, MO) in DMEM (Life Technologies) supplemented with fetal bovine serum (SAFC Bioscience, Lenexa, KS) for ten min. Cells were collected, quick-frozen in liquid nitrogen and stored at -85°C until vitamin A quantification by HPLC.

Statistical analysis. Data were expressed as means \pm standard deviation (SD). The statistical significance of differences was evaluated by unpaired Student's t-test for data in Figures 1C, 2, 5A and 5B, and by ANOVA combined with the Tukey test in Figure 6. P values of < 0.05 were considered to be statistically significant.

Results

Upregulation of Rbp2 and ApoA4 mRNAs in the intestines of SB rats. We examined the expression of mRNAs for genes related to retinoid absorption, transport and metabolism (Fig. 2). *Rbp1* mRNA was predominantly expressed in the liver and no significant changes between SB and sham rats were observed in either the liver or the intestine. The expression levels of *Rbp4* mRNAs in the ilea of SB and sham rats differed significantly ($P < 0.05$), though the expression of *Rbp4* mRNA in the intestines was considerably lower than in the livers. *Rbp2* mRNA was specifically expressed in the intestines, and the expression levels of *Rbp2* mRNA in SB rats were higher than in shams in both the jejunum and the ilea ($P < 0.05$). *Lrat* mRNA coding for retinoid-esterifying enzyme was expressed in both the liver and the intestine and did not show any significant differences between SB and sham animals. Intestinal expression levels of *ApoA4* mRNA in SB rats were higher than in shams in both the jejunum and the ilea ($P < 0.05$). Jejunal expression of *Fabp1* mRNA in SB rats was higher than in shams ($P < 0.05$).

Colocalization of CRBP II and LRAT in the intestinal absorptive epithelial cells of SB rats. We analyzed the cellular locations of CRBP II and LRAT proteins in the small intestines of SB and sham rats. CRBP II was found mainly in absorptive epithelial cells in the ilea of SB rats (Fig. 3A and C, arrows), while weak staining was observed in the ilea of sham rats (Fig. 3F and H). Immunohistologic staining of LRAT revealed its presence in the absorptive epithelial cells and lamina propria mucosae (Fig. 3B, D, G and I); staining intensities seemed to be at the same level in SB and sham rats. In SB rats, absorptive epithelial cells were positive for both CRBP II and LRAT staining (Fig. 3C and D, arrows). Some fibroblast-like cells in the lamina propria mucosae seemed to be double-positive (Fig. 3C and D, closed arrowheads) and others to be positive only for LRAT (Fig. 3C and D, open arrowheads). No histological differences were observed between sham and SB intestines by H&E staining (Fig. 3E and J).

Subcellular locations of LRAT, CRBP I and CRBP II in the liver and the intestine of SB and sham rats. We then analyzed the subcellular distributions of retinoid metabolism-related gene products in the liver and the

ileum. CRBP II was expressed in the cytosol fraction of the ileum (Fig. 4A) but not in the liver (Fig. 4B). LRAT was expressed in both the liver and the ileum (Fig. 4A and B), localized in the microsomal fractions. CRBP I was mainly expressed in the liver (Fig. 4B); expression levels were higher in the cytosol than in the mitochondria and microsomal fractions.

Downregulation of retinol and REs in the intestine of SB rats. We next used HPLC to quantify retinol and REs in the livers and the intestines of SB and sham rats. The content of REs in the jejunum of SB rats was lower than that of shams (Fig. 5A, $P < 0.05$). The retinol content in the ileum of SB rats was lower than that of shams (Fig. 5B, $P < 0.05$). There were no significant differences in the contents of retinol and REs in the livers of SB and sham rats (Fig. 5A and B).

Upregulation of REs in cultured cells overexpressing CRBP II and LRAT. We asked whether CRBP II affected the amount of REs produced by LRAT. Thus, we quantified REs in HEK293T cells overexpressing CRBP II and/or LRAT. As shown in Fig. 6, overexpression of LRAT in HEK293T cells led to the accumulation of a large amount of REs in the cells within ten min of addition of retinol to the medium ($P < 0.05$). CRBP II overexpression enhanced the accumulation of REs in LRAT-transfected HEK293T cells ($P < 0.05$).

Discussion

In this study of SB syndrome, we used a rat model to show that intestinal expression levels of *Rbp2* mRNA were significantly higher than those in shams (Fig. 2). The esterification of vitamin A is regulated by the cooperative actions of LRAT and the CRBPs (18,19). In accord with this, we showed that overexpression of CRBP II and LRAT cooperatively increased the amount of REs in a HEK293T cell line (Fig. 6). However, we observed the reduction of vitamin A content in the intestine of SB rats (Fig. 5A, B). The expression levels of *Apoa4* mRNA in SB rat intestine was significantly higher than in sham animals (Fig. 2), as was previously reported by Rubin *et al.* (20). Apolipoprotein A-IV is a component of chylomicrons (21) in which REs are transported (22). Thus, the upregulation of *Apoa4* mRNA might lead to enhanced transport of vitamin A from the absorptive epithelial cells to the lymphatics, resulting in the reduction of vitamin A content in the intestine of SB rats. In spite of the 75% reduction of the surface area of the intestine, our quantitative analysis showed no significant differences in the contents of retinol or REs in the livers of SB and sham rats (Fig. 5A, B), the major storage organ for vitamin A. This may be explained by the functional adaptation of the SB rat intestine mediated by the upregulation of *Rbp2* and *Apoa4* mRNAs.

It is generally accepted that LRAT is present in the endoplasmic reticulum (ER) (23) and the CRBPs are in the cytosol (24), and our data confirmed those presumptions (Fig. 4A and B). Nevertheless, LRAT and CRBPs likely act cooperatively for vitamin A esterification (25). The N-terminal portion and/or the C-terminal portion of LRAT are inserted into the membranes of the ER, exposing remnant hydrophilic portions to the cytosol (26,27). There, CRBPs probably deliver vitamin A to the cytosolic portion of LRAT.

In immunohistological staining of the small intestines, absorptive epithelial cells were positive for both CRBP II and LRAT (Fig. 3C and D). Their overlapping presence in the rat intestines provides *in vivo* evidence for the cooperative action of these two proteins to increase absorption of vitamin A. Some fibroblast-like cells in the lamina propria mucosae also seemed to be positive for both CRBP II and LRAT. We recently reported that vitamin A was taken up and stored in cells of the lamina propria mucosae of rat intestine and transmission electron microscopic analysis revealed that these vitamin A-storing cells in the lamina propria resembled extrahepatic stellate cells (EHSCs) (28). We speculate that the CRBP II- and LRAT- positive cells in lamina

propria represent EHSCs. If that is the case, EHSCs express CRBP II, different from HSCs that express CRBP I. It would be intriguing to determine whether the differential expression of CRBPs in HSCs and EHSCs leads to functional differences between those cells.

SB syndrome is linked to defective absorption of many kinds of nutrients including carbohydrates, fats, amino acids and vitamins. The resultant malnutrition could lead to diseases of various organs. For example, we previously reported that 90% resection of rat small intestine caused insufficiency of citrulline, a precursor of arginine (29), leading to focal tubulointerstitial fibrosis in the kidney (30). Also, liver fibrosis occurred after massive small bowel resection in the neonate piglet SB model (31). The increased esterification and transport of retinol in the absorptive epithelial cells of the SB intestines, which is suggested in this study, may contribute to prevent such fibrosis, because suppressive effects of vitamin A on various kinds of fibrosis are known (32).

In conclusion, our results suggest that esterification and transport of vitamin A are enhanced in the absorptive epithelial cells of the SB rat intestines through the upregulation of *Rbp2* and *Apoa4* mRNAs.

Acknowledgements

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Figure Legends

Figure 1. Generation of the SB rat model. Schematic representations of SB and sham rat models are shown (A). The anastomosed part of the intestine (arrow) and the ileocecal junction (white arrowhead) are shown (B). Body weight changes of SB and sham rats were recorded every day after the operation for one week (C). Data were expressed as means \pm SD. P values of < 0.05 were considered to be statistically significant and are shown by asterisks.

Figure 2. Quantitative analysis of mRNA levels in SB and sham rats. The mRNAs of *Rbp1*, *Rbp2*, *Rbp4*, *Lrat*, *Apoa4*, *Apob*, *Fabp1* and *Fabp2* were assessed using quantitative RT-PCR. Data were expressed as means \pm SD. P values of < 0.05 were considered to be statistically significant and are shown by asterisks.

Figure 3. Cellular localizations of CRBP II and LRAT. Immunohistological staining of serial sections of SB rat ileum (A-D) and sham rat ileum (F-I) was performed using antibodies against CRBP II (A, C, F and H) or LRAT (B, D, G and I). High-magnification images of rectangles in A, B, F and G are shown in C, D, H and I, respectively. HE-stained sections were shown (E, J). Arrows: absorptive epithelial cells positive for both CRBP II and LRAT. Closed arrowheads: fibroblast-like cells in lamina propria mucosae that were positive for both CRBP II and LRAT. Open arrowheads: fibroblast-like cells positive for LRAT and negative for CRBP II. Scale bar in G is 100 μ m and applies to A, B, E, F and J. Scale bar in I is 20 μ m and applies to C, D and H.

Figure 4. Subcellular localizations of CRBP II and LRAT. Subcellular fractions of tissues from ilea (A) and livers (B) from SB and sham rats were analyzed by Western blotting using antibodies against LRAT, CRBP I and CRBP II.

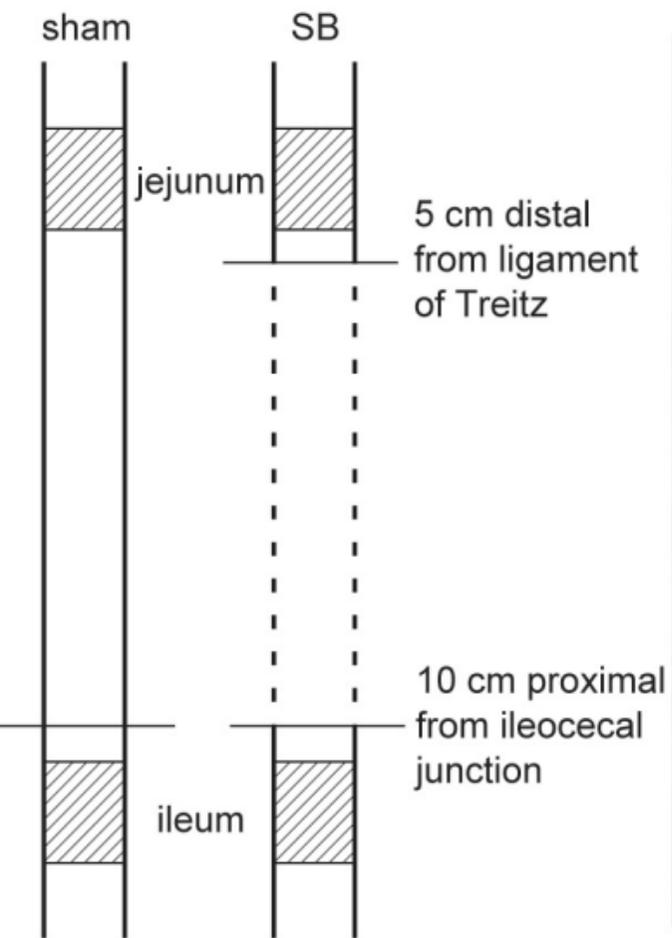
Figure 5. Quantitative analysis of retinol and REs in livers and intestines of SB and sham rats. The REs (A) and retinol (B) in the livers and the intestines of SB and sham rats were quantified by HPLC. Data were

expressed as means \pm SD. P values < 0.05 were considered to be statistically significant and are shown by asterisks.

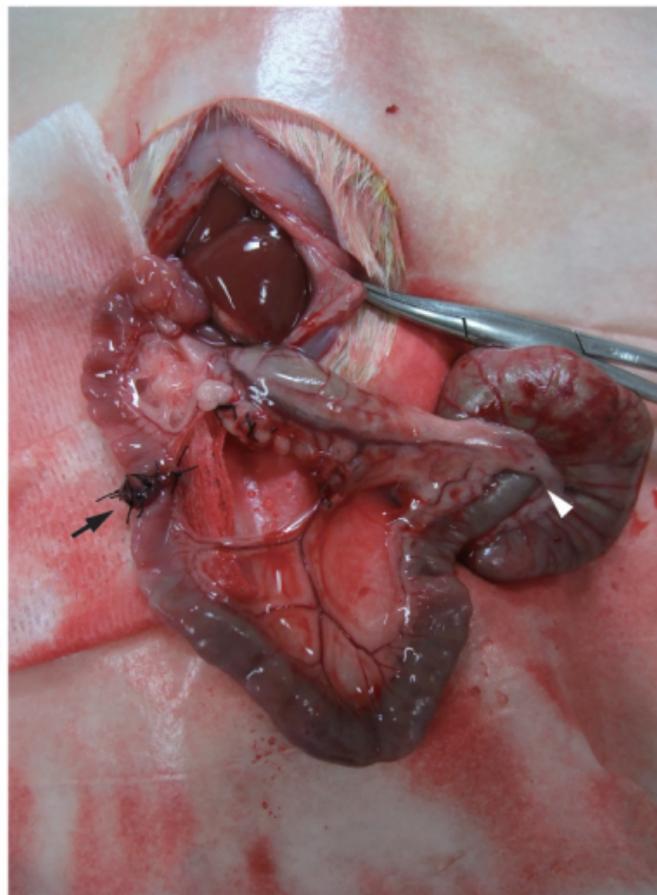
Figure 6. Quantification of REs in cultured cells overexpressing CRBP II and/or LRAT. HEK293T cells were transfected with vectors containing cDNAs for CRBP II and/or LRAT. Eighteen h after transfection, cells were treated with 10 μ M retinol and further cultured for ten min. Cells were collected and REs were quantified using HPLC. Data were expressed as means \pm SD. P values < 0.05 were considered to be statistically significant and are shown by asterisks.

Table 1. Primers for RT-PCR.

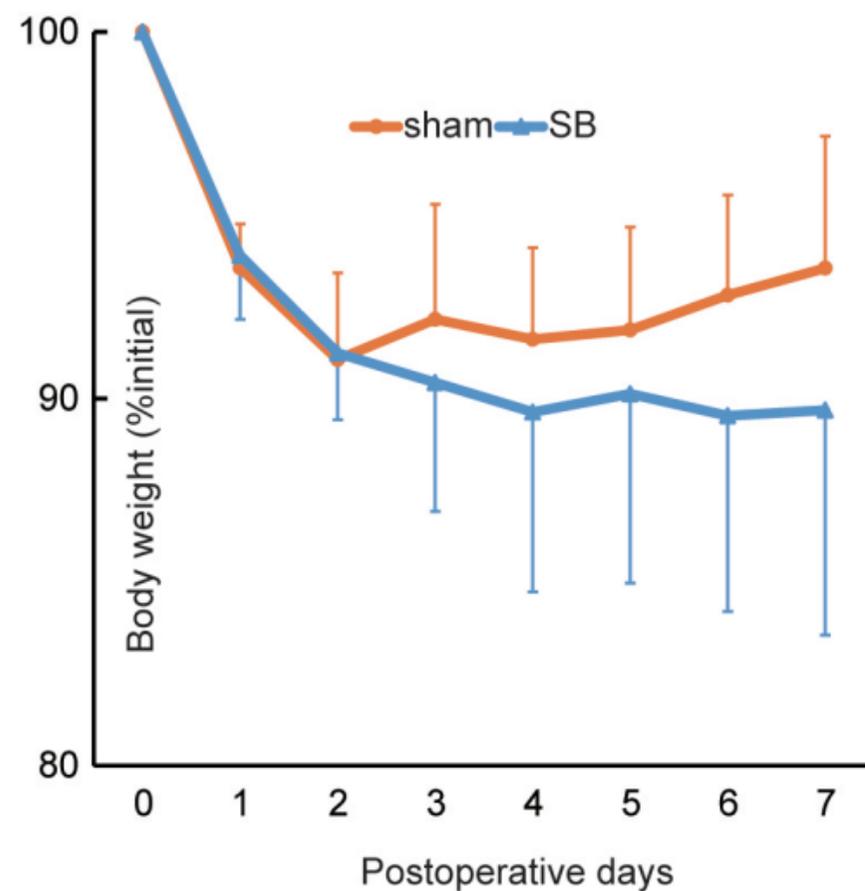
Gene	Forward	Reverse	Length
<i>Gapdh</i>	ACAGCAACTCCCATTCTTCC	TCCACCACCCTGTTGCTGTA	118 bp
<i>Rbp1</i>	AGGCATAGATGACCGCAAGT	TCATCACCTCAATCCACTG	117 bp
<i>Rbp2</i>	GAAACACCCTGGTGTGCGTG	GAACACTTGTCGACACACCT	122 bp
<i>Rbp4</i>	GTTTTCTCGTGACCCCAATG	ACTGTTTCTTGAGGGTCTGC	139 bp
<i>Lrat</i>	GCGAACACTTTGTGACCTAC	GACAGCTGAAGCAAGACAAC	119 bp
<i>Apoa4</i>	TGAAGGCTGTGGTGCTGA	CCTCCTGGCATTGTTGC	126 bp
<i>Apob</i>	CAGCCATAGGCACTGTGAGTC	TGTCCTCCACTCCATTTTG	126 bp
<i>Fabp1</i>	TTCTCCGGCAAGTACCAAGT	TTCATGCACGATTTCTGACAC	126 bp
<i>Fabp2</i>	TGATGGCACTTGAAAGTAGAC	CCTTCCTGTGTGATCGTCAG	126 bp



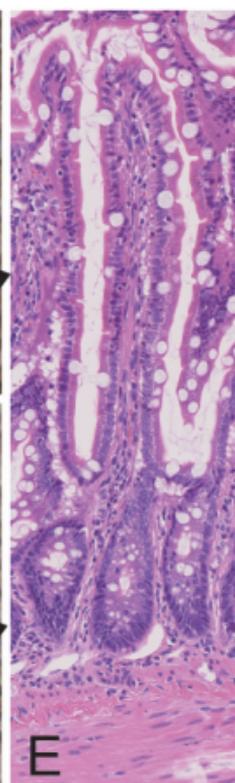
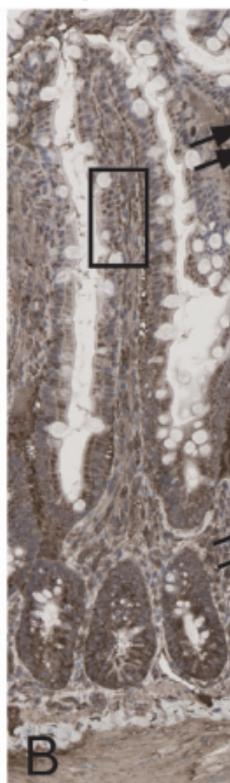
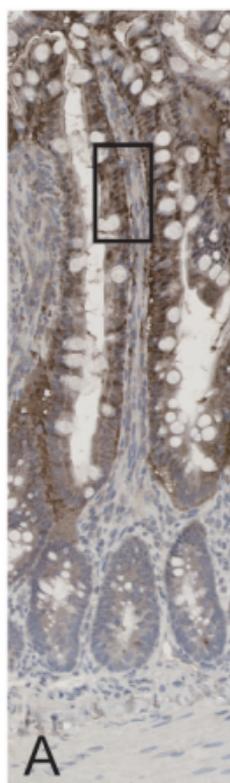
B



C



SB



sham

